Potential Nitrification Activity Reflects Ammonia Oxidising Bacteria but not Archaea activity across a Soil-Sediment Gradient

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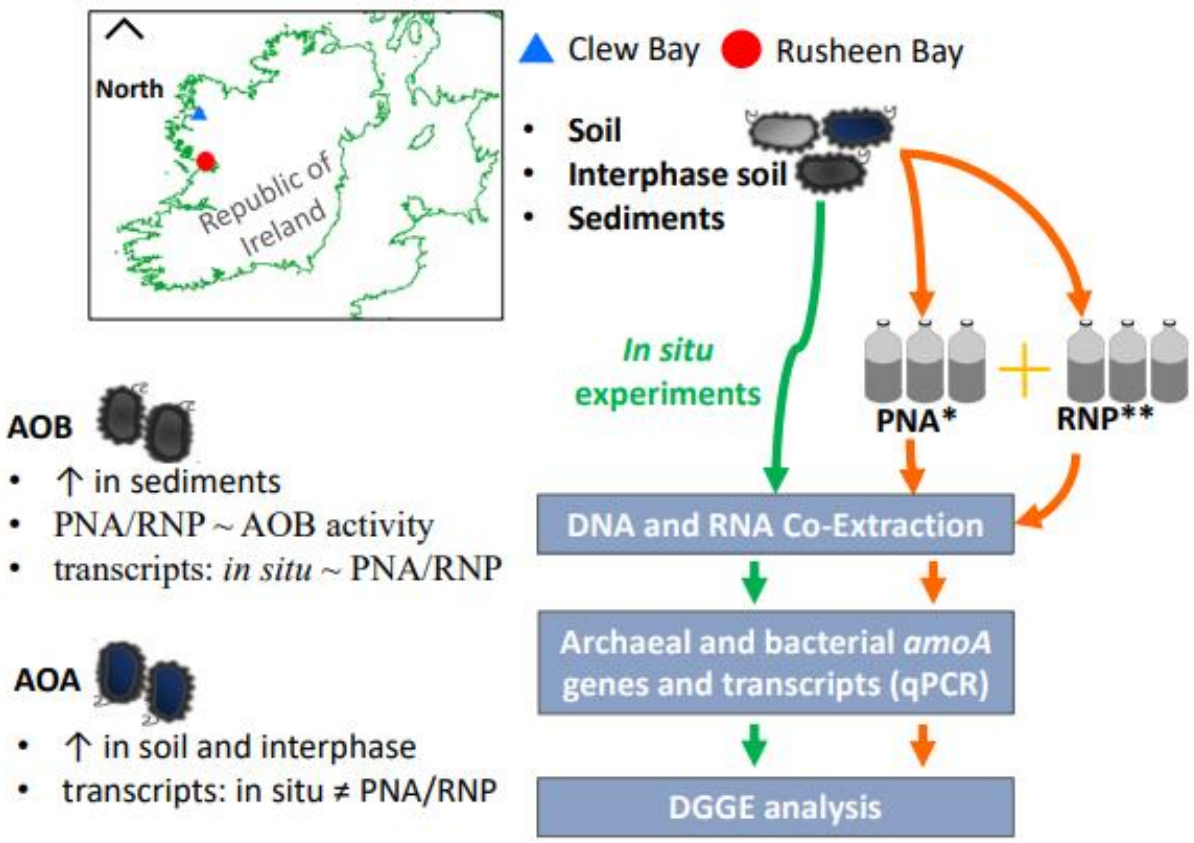
Abstract

Terrestrial-marine ecosystems have important ecologically relevant roles influencing the retention and mobility of nitrogen entering coastal ecosystems. The sharp physico-chemical gradients represent an ideal environment to elucidate the relative contributions of ammonia oxidizing archaea (AOA) and bacteria (AOB) to the nitrification process. Here we examined the activity of ammonia oxidisers (AO) across two coastal bays soil-sediment gradients to explore the functional shift from AOA to AOB, and determine if transcriptional activity within the environment (*in situ*)

was emulated in laboratory potential nitrification activity incubations. To do this gene and transcripts abundance and diversity were measured along with potential nitrification activity (PNA) and recovery nitrification potential (RNP) from a series of soil, interface and sediment sites. We compared the composition of *amoA* transcript community structure *in situ* vs. PNA/RNP to see if the active AOA and AOB were similar in the environment and in the laboratory experiments. AOA was dominant at gene and transcript level in soil and interphase sites, but active transcripts *in situ* did not match those within the PNA/RNP assays. AOB was dominant at gene and transcript level in sediments and here transcripts *in situ* and within the PNA/RNP were similar. A high correlation between AOB transcripts and PNA in sediments was observed but a negative correlation for AOA in soils was seen. Our data indicates that while the PNA/RNP may be a good proxy for AOB activity in these sediments, it was not for AOA dominated soil due to unfavorable incubation conditions.

Graphical Abstract



Introduction

Nitrogen (N) is an essential component of all living organisms and a significant controller of primary production in marine and terrestrial environments. The N cycle is a complex combination of assimilatory and dissimilatory biogeochemical pathways driven mainly by microorganisms (Stein and Klotz, 2016). Nitrification plays a central role, oxidising ammonia to nitrite and nitrate making nitrate/nitrite available for reduction and removal from the ecosystem by denitrification. Nitrification is also directly linked to the carbon (C) cycle via autotrophic fixation of CO_2 (Gruber and Galloway, 2008). Furthermore, it has been shown to be capable of generating notable amounts of the noxious greenhouse gas N_2O via incomplete oxidation of hydroxylamine and/or nitrifier denitrification (Yang et al., 2017).

Nitrification has long been defined as an aerobic two-step reaction carried out by both ammonia oxidisers (AO), who convert ammonia (NH_4^+) to nitrite (NO_2^-), and nitrite oxidizing bacteria (NOB), who convert NO_2^- to nitrate (NO_3^-). AO includes both ammonia oxidizing bacteria (β- and γ-proteobacteria; AOB) and ammonia oxidizing archaea (*Thaumarchaea*; AOA). Recently, the classical view of nitrification, conducted by separate AO and NOB has been challenged by the discovery of bacteria capable of complete oxidation of ammonium to nitrate (COMAMMOX). Researchers have recently provided evidence that *Nitrospira spp*., a genus previously considered involved only in nitrite oxidation, is capable of complete oxidation of NH_4^+ to NO_3^- (Daims et al., 2015; van Kessel et al. 2015).

Ammonia oxidation has received significant attention in the literature and numerous studies have been conducted to investigate the contribution of AOA and AOB to nitrification across different natural and engineered ecosystems (e.g. Duff et al 2017; Fernandes et al., 2016; Gubry-Rangin et

al., 2010; Jäntti et al., 2018; Liu et al., 2013; Muukhtar et al., 2019; Yi et al., 2020; Zhang et al 2018;). A general understanding of the distribution and dominance of AOA and AOB has emerged with AOB dominant in ammonia-rich coastal areas and estuaries (Duff et al., 2017; Lisa et al., 2015; Mosier and Francis, 2008), wastewater (Bai et al., 2012) and N-rich grassland soils (Di et al., 2009). AOA are more abundant in low-ammonium environments, such as ocean waters, river sediment, freshwater lakes, and rice paddy soils (Li et al., 2015; Liu et al., 2013; Santos et al., 2018) with pH as a further driver of niche differentiation in soils (Gubry-Rangin et al., 2010). However, more recently AOAs with similar Km to *Nitrosomonas* have been isolated from soil (Kits et al 2017; Hink et al., 2017). This, taken together with reports of AOA dominance in high-ammonia estuaries (Moin et al., 2009; Cao et al., 2011), indicates that ammonia concentration alone does not explain AO dominance and other factors such as pH (Gubry-Rangin et al., 2010) and/or the source of ammonia nitrogen (organic vs. inorganic) may also play a role (Aigle et al., 2020; Di et al., 2010).

In general, the relative contribution of AOB and AOA in the environment is determined by quantification of *amoA* genes at DNA level, while nitrifier activity can be determined using ^{15}N isotopic approaches to measure *in situ* rates (Rysgaard et al., 1993; Hart et al., 1996). ^{15}N experiments tend to be time consuming and require access to specialized isotope ratio mass spectrometry facilities. Alternatively, potential nitrification activity (PNA), is a simple, cheap and fast approach that has been widely used in soil science (for a critical review see Hazard et al., 2021) and to a lesser extent in coastal sediments (Li et al., 2014; Duff et al., 2017, Zhang et al., 2019). PNA is not an *in situ* rate measurement, but a measure of the maximum capacity of a soil or sediments nitrifying community to oxidise ammonia to nitrate under a given set of laboratory conditions. Recovery of nitrification potential (RNP) is an extension of PNA (Taylor et al., 2010).

In RNP acetylene (C_2H_2) irreversibly stops the synthesis of the ammonia monooxygenase (AMO) enzyme (Hyman and Wood, 1985), but upon removal, ammonia oxidation can resume the synthesis of new AMO enzyme via transcription and translation.

However, there are some limitations to both *amoA* gene quantification and PNA. First, gene abundance does not imply activity. Second, theoretically PNA/RNP reflects the maximum nitrification/recovery rate of the community to an amendment of ammonia, it does not inform of the individual contribution of AOB and AOA, nor if the organisms responding to the assay reflect the *in situ* active organisms. Moreover, PNA is likely a biased measurement (Santoro et al., 2010) which favors AOB due to the ammonia concentration supplied (100 µM to 1 mM) (Duff et al., 2017; Liu et al., 2013; Walkup et al., 2020). From enrichment cultures, it has been shown that the growth rate of AOB is significantly reduced at NH_4^+ concentration between 39 µM and 50 mM (Koper et al., 2010; Park et al., 2009), while AOAs growth rate is lower at NH_4^+ concentrations between 2 µM and 2 mM (Koper et al., 2010). Therefore, PNA incubations do not inform what organisms are responding to the assay, if these reflect the active AOs in the environment, and if PNA is better suited to reflect AOB than AOA activity.

In this study, we explore the response of AOA and AOB to PNA and RNP across the terrestrial-marine environment where we have previously shown the differing contribution of AOA and AOB (Zhang et al., 2018). This work indicated that AOA were driving ammonia oxidation in soil and AOB in sediments. This gradient provides an ideal site to disentangle the relationship between gene and transcript abundance and PNA/RNP for AOB and AOA, respectively. We aimed to understand the extent to which *amoA* gene and transcript abundance reflected PNA/RNP as a proxy for AOB and AOA activity. We confirm the contribution of AOA and AOB across the soil and sediment gradients via a series of PNA and RNP incubations with selective inhibitors combined

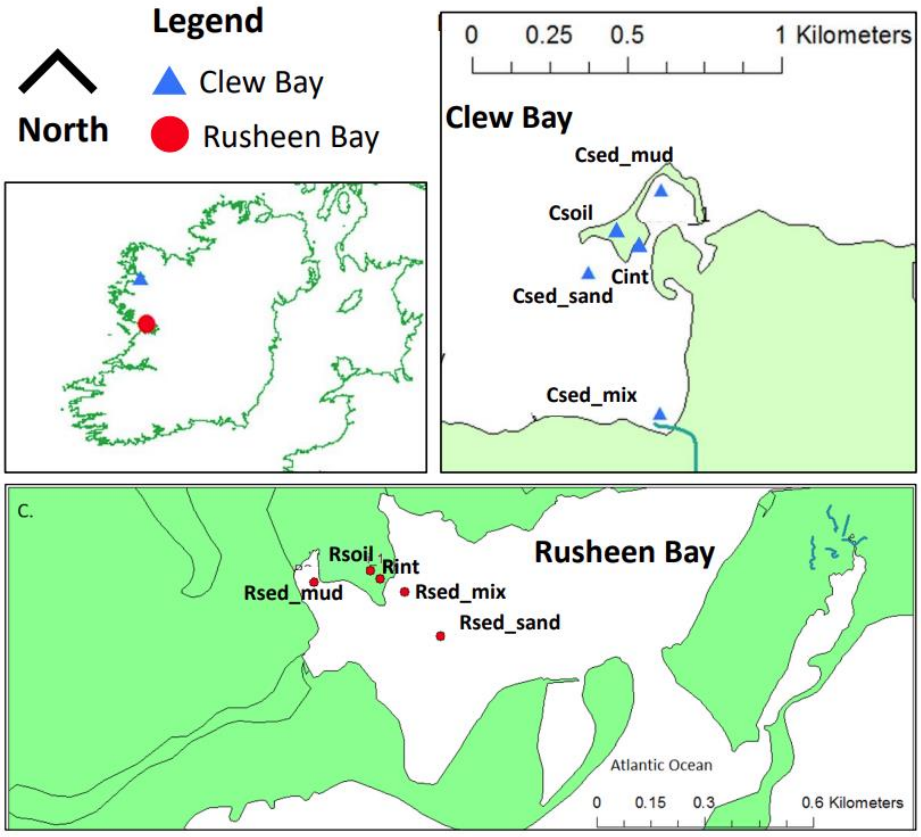
with quantification of the respective AOA and AOB *amoA* transcripts. Then using this gradient, we explored the contribution of AOA and AOB to PNA and RNP, hypothesising that PNA activity better reflects *in situ* active AOB than AOA.

Materials and Methods

Sites Descriptions and Sampling

The study was carried out in two small (~ 1 km^2) intertidal coastal bays - Rusheen and Clew bay located on the west coast of the Republic of Ireland (**Fig. 1**). Rusheen bay (53° 25.5894'N, -9° 11.9532'W) is sheltered by a beach and is an intertidal mud/sand flat, situated along the north side of Galway bay on the edge of Galway city (population 75000). Clew bay (53° 78.6962'N, -9° 64.9515'W) is an intertidal mud/sand flat situated in a rural area surrounded by agricultural land, mainly sheep farming, approximately 120 km north-west of Rusheen Bay. A beach shelters the bay on its seaward side. Numerous groundwater upwelling and freshwater streams are present in the bay. Soil and sediment samples were collected in February 2015. In both bays, samples were collected from five different points along a soil-benthic gradient based on previously performed granulometric analysis (Duff et al., 2017; Zhang et al., 2018) (**Fig. 1**). The gradient originated near the sea (i.e., 30 m) in vegetated soil (Rsoil and Csoil). It continued towards the water edge, into a grass-covered interface zone where high tides occasionally submerge the soil with seawater (Rint and Cint). Interface soils were water-logged and displayed signs of coastal erosion. The gradient ended in coastal sediments of the intertidal zone characterized by different sand:mud ratio with sediments characterized by a) high composition of mud (Rsed_mud and Csed_mud), b) a mix of

126 mud and sand (Rsed_mix and Csed_mix), c) sand only (Rsed_sand and Csed_sand) (Duff et al.,
127 2017; Zhang et al., 2018).



128

129 **Figure 1.** (A) Sampling locations are on the west coast of Ireland. Maps of Clew (B) and Rusheen
130 bays (C) depict the location of sampling sites along the soil-sediment gradients.

131 Soil and intertidal sediments (top 0-5 cm) were collected at low tide on February 23rd and 27th
132 2015 from Rusheen and Clew bay, respectively. Samples from ten random points within a 10 m^2
133 area were pooled as one replicate with three such replicates collected from each 10 m^2 sample
134 location (**Fig. 1**). Replicates were homogenized, subsampled into 0.5 g aliquots, flash frozen and
135 then stored at -80°C for subsequent molecular analysis. These samples are referred to as the *in situ*
136 samples. Soil and sediment samples for PNA/RNP and physicochemical analysis were returned to

137 the lab on ice and stored at 4°C until analysis (performed not later than 6h from sampling). Soil
138 was manually broken up, removing stones and roots, and homogenized prior to PNA/RNP and
139 physico-chemical analysis.

140 **Determination of Physico-chemical Parameters**

141 Soil and sediment temperature was measured at each site with a mercury thermometer. Salinity of
142 site seawater was determined with a refractometer (Coral Farm, Ireland) and reported using with
143 the Practical Salinity Scale. Soil salinity was measured following extraction of pore-water after
144 centrifugation. pH was determined by mixing soil and sediments with deionised water at a ratio of
145 1:2 and measured with an Orion pH meter, model 420A (Cole Parmer, Ireland). Water content was
146 calculated via the weight loss of a known amount of sample dried at 105°C until a constant value
147 was reached (e.g., typically 24 h). Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were
148 extracted from the sediment by incubating 5 g fresh weight (FW) sediment with 30 ml of 1 M KCl
149 for 1 h at 15°C on a shaker. Samples were then filtered with grade 52 Whatmann filter paper
150 (Fisher scientific, Ireland) and frozen at -20°C until further analysis. Dissolved inorganic nitrogen
151 (i.e., NO_2^-, NO_2^-+NO_3^- and NH_4^+) was measured using colorimetric methods on a plate reader as
152 described by Bollmann et al. (2011). Dissolved organic carbon and nitrogen (DOC/DON) were
153 analysed from acidified samples on a Shimadzu TOC-L with TNM-L module. Limits of detection
154 are provided in supplementary information (Tab. S2).

155 **PNA, RNP and use of Selective Inhibitors**

156 PNAs were carried out in triplicate at all sites in both bays (**Fig. 1**). A schematic of the PNA and
157 RNP experiments can be found in **Tab. S1**. Five g of fresh weight (FW) soil or sediment was added
158 to 250 ml glass bottle (Voight, Kansas, USA) containing 30 ml of either phosphate-buffered saline

(PBS) 0.1 M pH 7.1 for soil and interface samples, (salinity adjusted to *in situ* values) or 0.2 μm-filtered site water for sediments. All were amended with 24 μM sodium azide, an inhibitor of nitrite oxidation (NaN_3, nitrite oxidation inhibitor; Sigma, Ireland; Ginestet et al., 1998) and 250 μM NH_4^+ [$(NH_4)_2SO_4$, Sigma, Ireland]. These conditions, in the absence of selective AO inhibitors, were considered the standard PNA. Two additional PNA assays were set up to investigate the contribution of ammonia oxidizing bacteria and archaea: a) PNA amended with 50 mM of allylthiourea (ATU, Sigma, Ireland), as a selective inhibitor of the bacterial ammonia monoxygenase, sequestering the Cu^{2+} ions (Wang et al., 2017); b) PNA amended with 75 μM of cycloheximide (CHX, Sigma, Ireland), a eukaryote and archaeal inhibitor of protein synthesis (Vajrala et al., 2014; Wright et al., 2020). However, there are notable limitations to each inhibitor, with some AOA sensitive to ATU (Lehtovirta-Morley et al., 2013; Shen et al., 2013) and some AOA insensitive to CHX (Taylor et al, 2010). Therefore, we refer to ATU-sensitive and CHX-sensitive ammonia oxidisers, as illustrated in **Fig. S1**. Soil and sediment slurries were incubated in open bottles in the dark on a shaker at 90 rpm at 15°C for 48 h. Preliminary experiments showed that these conditions were optimal for the measured rates, with aeration preventing N immobilization and denitrification (data not shown). Background concentrations of NO_2^- were measured after 10 mins of incubation. At the end of the experiments, NO_2^- was extracted with two volumes of 1 M KCl following 30 min agitation at 90 rpm. Nitrite was measured as described above. At the end of the PNA incubation, 0.5 g of soil/sediment slurry was collected from each bottle in 2 ml RNAse-free screw cap tubes and immediately flash-frozen in liquid N. Samples were stored at -80°C upon DNA/RNA extraction.

RNPs were carried out at all sites in both bays as described by Taylor et al. (2010). Briefly, 5 g of soil/wet sediments were added to 250 ml glass bottle containing 30 ml of PBS/site water amended

with 24 μM NaN_3. Bottles were closed by butyl stoppers and 0.1% headspace acetylene gas (C_2H_2) injected with a sterile syringe. C_2H_2 is an effective inhibitor of nitrification irreversibly blocking archaeal and bacterial ammonia monooxygenase (AMO) enzyme. Bottles were incubated in the dark for 3 h, after preliminary experiments showed this was sufficient time to inhibit the ammonia monooxygenase (data not shown). C_2H_2 was removed by degassing for 10 min using a vacuum pump. Butyl stoppers were removed to permit aeration and 250 μM NH_4^+ was added. Bottles were then incubated for 72h (24 h to allow the synthesis of new AMO followed by a 48-h incubation). Preliminary experiments showed that these conditions allowed efficient blocking and recovery with high nitrification rates (data not shown). These settings in the absence of inhibitors were considered the standard RNP. For all sites, two additional incubations were included: a) RNP with 50 μM ATU and 250 μM NH_4^+.; b) RNP with 75 μM CHX and 250 μM NH_4 (see supplementary Fig. S1). At the end of the 72 h incubation experiments, NO_2^- was extracted and measured as indicated above. 0.5 g of soil/sediment slurry were collected from each bottle in 2 ml screw cap tubes and immediately flash-frozen in liquid N for subsequent molecular analysis.

DNA and RNA Co-Extraction

DNA and RNA extractions from soils, sediments and soil/sediment slurries were carried out as described by Tatti et al. (2016). Briefly, 0.5-0.7 g of fresh soil or sediment and 0.5 g soil/sediment slurry were added to Lysing Matrix E tubes with 500 µl CTAB/Phosphate buffer and 500 µl Phenol:Chloroform:Isoamyl alcohol (25:24:1) and vortexed at full speed for 2.5 minutes, followed by centrifugation. The top layer was removed and added to 500 µl of Chloroform:Isoamyl alcohol (24:1), mixed, and centrifuged. This was repeated, and the top layer was removed, and DNA/RNA precipitated with two volumes of 30% PEG/1.6 M NaCl. Nucleic acids were pelleted by centrifugation and re-suspended in 50 µl DEPC (diethylpyrocarbonate) water. A 30 µl aliquot of

DNA/RNA was removed to prepare RNA using TURBO DNase (Ambion, UK) according to the manufacturer's instructions. The absence of DNA from the RNA fraction was confirmed by no amplification of the 16S rRNA gene using primers F63 (5'-CAG GCC TAA CAC ATG GCA AGT C-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Marchesi et al., 1998; Muyzer et al., 1993). 2 µl of RNA template (undiluted, 10^{-1} or 10^{-2} dilution) were added to a 50 µl PCR mixture containing 5 µl PCR buffer including $MgCl_2$ (Sigma Aldrich, Ireland), 0.2 mM of each deoxynucleoside triphosphate (dNTP; Ambion, UK), 0.25 µM of each primer (Eurofins, Germany), and 2.5 units of *Taq* polymerase (Sigma Aldrich, Ireland). The reaction was initially denatured at 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min; followed by a final extension step at 72°C for 7 min. The absence of a 16S rRNA gene band was visually confirmed on a 1% agarose gel. DNA and RNA were quantified using Quant-iT™ DNA and RNA Assay Kits (ThermoFisher, UK) and Qubit fluorometer (Thermofisher, UK). DNA and RNA were stored at -80°C until further analysis.

cDNA Synthesis

RNA was converted to cDNA using Superscript III (Life Science, USA). Gene specific reverse transcription PCR (RT-PCR) amplification was performed on RNA targeting AOA and AOB *amoA* transcripts. The initial RT reaction mixture contained 250 ng of RNA, 2 mM of the appropriate reverse primer, archaeal amoA616R (GCC ATC CAT CTG TAT GTC CA, Tourna et al., 2008) or bacterial amoA2R (CCC CTC KGS AAA GCC TTC TTC, Rotthauwe et al., 1997), and 10 mM of each dNTP. The mixture was denatured at 65°C for 5 min and transferred to ice for 1 min. 5X first-strand buffer, 0.1 mM RNAse Out (Thermo Fisher, UK), and 200 units SuperScript III was added to the reaction mixture and incubated at 55°C for 50 min, followed by inactivation of the reaction at 72°C for 10 min. Samples were stored at -80°C until further analysis.

Quantitative PCR

Archaeal and bacterial *amoA* genes (i.e., DNA) and transcripts (i.e., cDNA) were quantified by qPCR from triplicate samples collected along the terrestrial-marine gradients. Triplicate no-template controls (NTC) and appropriate standard curve were included in each assay. DNA/cDNA extracts were tested for the presence of co-extracted inhibitory substances, as previously described (Tatti et al., 2016). A 1:10 dilution was used to remove inhibitory substances (data not shown). Quantitative PCR was performed using the SsoFast™ EvaGreen® master mix (Biorad, England), archaeal a*moA* primers amoA23F (ATG GTC TGG CTW AGA CG) (Könneke et al., 2005) and amoA616R (Tourna et al., 2008) at 200 nM each or bacterial *amoA* primers amoA1F (GGG GHT TYT ACT GGT GGT) (Stephen et al., 1999) and amoA2R (CCC CTC BGS AAA VCC TTC TTC) (Hornek et al., 2006) at 200 nM each, plus 1 ng of DNA/cDNA in a 20 µl reaction. These AOB primers were selected for their higher coverage of AOB *amoA* (Hornek et al., 2006; Stephen et al 1999). Cycling conditions were: 1 cycle of 95°C for 5 min and then 40 cycles of 95°C for 30 s, 47°C AOB/ 55 °C AOA for 30 s, 72°C for 60 s. Fluorescence was measured at 82°C AOB/81°C AOA. qPCR assays were conducted using Roche Lightcycler 480 (Roche). DNA and RNA standard curves were constructed by amplifying the gene of interest according to Smith et al. 2006. The standard curve descriptors for the abundance of nitrifiers communities were: for archaeal *amoA* gene copy numbers/transcripts: slope = -2.97, Efficiency = 117.1%, R^2 = 0.996; for bacteria *amoA* gene copy numbers/transcripts: slope = -3.03, Efficiency = 112.8%, R^2 = 0.993. Successful amplification of the desired fragments was assessed by agarose gel, while specificity was checked by melt curve analysis.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

PCR for DGGE analysis was carried out on archaeal and bacterial *amoA* at DNA and cDNA levels for all samples along the terrestrial-benthic gradient for both *in situ* and *in vitro* (PNA and RNP assays). *amoA* genes were PCR-amplified for DGGE using 0.25 μM amoA23f/amoA616r and amoA1F/amoA2R primers without GC tail for archaea and bacteria, respectively and 2 U Taq Polymerase (Sigma, Ireland). PCR conditions were as follows: 94°C for 3 min; 10X (94°C for 30 s; 62°C (AOB)/60°C (AOA) for 45 s; -0.5°C at 3°/s; 72°C for 1 min) 30 X (94°C for 30 s; 57°C (AOB)/55°C (AOA) for 45 s; 72°C for 1 min) with a final elongation step 72°C for 10 min. Correct size amplicons were checked by gel electrophoresis. DGGE was performed only on Rusheen bay samples, as Clew bay cDNA samples did not lead to a satisfactory yield of amplicons for DGGE. DGGE analysis was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories). PCR products were loaded onto a 6% polyacrylamide gel containing a 15-45% or 20-45% denaturant, for archaeal and bacterial *amoA,* respectively. The gel was run at 70 V for 15 h with a constant temperature of 60°C. The gel was removed from the electrophoresis tank and stained with GelStar™ Nucleic Acid Gel Stain 10,000X (Fisher Scientific, Ireland) for 30 min and washed in 1X TAE buffer for 5 min before taking the final picture under 300 nm UV light. Bands exhibiting presence/absence or contrasting relative intensities between DGGE profiles of genes and transcripts were excised from DGGE profiles. DNA bands were placed in water overnight at -20°C and then used as templates for PCR targeting archaeal or bacterial *amoA*. Cloning of PCR products was carried out using pGEM T-Easy vector and competent *Escherichia coli* JM109 cells (Promega, Ireland). Clones obtained from each excised band excised were randomly selected and subjected to PCR. A total of 25 bands were excised, 8 for archaeal and 17 for bacterial *amoA* and sent for Sanger sequencing at Source Bioscience (Dublin, Ireland).

Clone sequences showing ≥99% similarity were considered the same (Acinas et al., 2005). Sequences were compared to sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/) and were then imported into Mega7 (Kumar et al., 2016) aligned using Clustal W. Phylogenetic trees were constructed using the neighbor-joining method, and tree topology was evaluated by bootstrap analysis. Sequences generated in this study have been deposited in the GenBank database (Accession Numbers: MW369505 to MW369529).

Statistical Analysis

Graphpad PRISM was used to conduct statistical analyses (Graphpad Software Inc., La Jolla, USA). Non-normal data were log-transformed. General linear model analysis of variance was performed based on a completely randomized design with site, or site and microbial group (i.e., AOA/AOB), or site and potential plus ATU/CHX as fixed factor(s), for the physico-chemical gradient characteristics, DNA/cDNA *amoA* qPCR quantification and PNA/RNP analysis, respectively. Site, microbial group and potential plus ATU/CHX means were compared by a post hoc Tukey honesty (HSD) test. Pearson's test (R^2 coefficient) was used to determine possible linear relationships between the parameters: pH, NO_3, NH_4^+, DON, DOC, PNA, RNP, pH, archaeal and bacterial *amoA* abundance and transcript levels and were considered significant from a two-tailed test at $P<0.05$.

Phoretix software (TotalLab Ltd, Newcastle-Upon-Tyne, UK) was used to obtain matrices from DGGE profiles consisting of the relative intensity of each band (*i.e.*, ratios of the intensity of each band to the total band intensity). Similarities between the banding patterns generated by PCR-DGGE of selected samples were analysed using Pearsons correlation coefficient and were

displayed as a dendrogram. Clustering algorithms were used to calculate the unweighted pair group method with arithmetic averages (UPGMA).

Matrices were analysed using PRIMER v6 software (Plymouth, UK). Rank similarity matrices were computed. The difference between *in situ* (DNA/RNA extracted from environmental samples) and *in vitro* (DNA/RNA extracted from PNA/RNP samples) of bacterial and archaeal ammonia oxidizing community structures was tested by Permutational Multivariate Analysis of Variance (PERMANOVA).

Results

Physico-Chemical Characteristics of the Soil-Sediment Gradient

The two-chosen soil-sediment gradients showed similar chemical-physical characteristics (**Tab. 1**). Both bays presented an increasing gradient in pH from the soil to the marine site. In Rusheen bay, the pH went from 7.2 in soil (e.g., Rsoil_1) to 8.5 in marine sandy site (Rsed_sand). In Clew bay, the soil pH was slightly acidic (6.4 in Csoil) and then gradually increased to 8.9 in the marine sandy site (Csed_sand). Similarly, salinity increased along the two gradients, ranging from 4.9-5.8 in the soil sites to 33.0-34.0 in marine sediments. In Clew bay, due to the input of freshwater from a small natural stream, the gravelly sandy-muddy site (Csed_mix) had a salinity of 8.2 at the time of sampling (low tide). Both bays generally showed low NO_3^- concentrations, ranging from 0.40± 0.14 to 2.26±SD0.09 µg N g^{-1} soil/sediment. The muddy sites (Rsed_mud and Csed_mud) had the highest values, while the sandy sites (Rsed_sand and Csed_mix) showed the lowest NO_3^- concentration in each bay. Interestingly, NO_2^- concentration in Clew bay were higher than Rusheen bay, where they ranged from 0.25±0.04 (Csed_sand) to 2.05±1.52 (Csed_mud) µg N g^{-1} soil/sediment and were greater than NO_3^- concentrations in Cint and Csed_mix (1.82±1.50) to

315 2.05±1.52 µg N g^{-1} soil/sediment, respectively). In contrast NO_2^- was lower in Rusheen bay
316 ranging from 0.12±0.04 to 0.38±0.23 µg N g^{-1} soil/sediment and always lower than the
317 corresponding site NO_3^-. The two bays showed different NH_4^+, DOC and DON trends along their
318 soil-marine sites (**Tab. 1**). In Rusheen bay, NH_4^+ concentration ranged from 52.75±3.79 to
319 119.88±35.57 µg N g^{-1} soil/sediment with the interface site (Rint) having the highest NH_4^+ values,
320 while the sandy site (Rsed_sand) showed the lowest NH_4^+ concentration. In Clew bay, the NH_4^+
321 concentration ranged from 33.55±8.06 to 118.42±65.73 µg N g^{-1} soil/sediment, with the soil site
322 (Csoil) showing the lowest values and the interface site (Cint) having the highest. In Rusheen bay,
323 DOC concentration ranged from 60.4±0.97 to 92.55±3.12 µg C g^{-1} soil/sediment and in Clew bay
324 from 51.61±5.40 to 149.17±1.56.

325 DON concentration ranged from 10.68±1.01 to 17.67±0.73 µg N g^{-1} soil/sediment in Rusheen bay,
326 and from 11.5±3.01 to 24.3±1.96 µg N g^{-1} soil/sediment in Clew bay. In Rusheen bay, soil, and
327 interface sites (Rsoil and Rint) showed the highest DOC/DON concentrations, while the marine
328 sandy site (Rsed_sand) had the lowest. In Clew bay, the muddy sediment site (Csed_mud) showed
329 the highest DOC/DON values, while the lowest concentrations of DOC/DON were measured in
330 soil and interface sites (Csoil and Cint).

331 **Abundance of Archaeal/Bacterial *amoA* Genes and Transcripts along the Gradient**

332 *amoA* genes were quantified from all sites. AOB ranged from 2.76 x 10^5 ± 2.67 x 10^5 Csoi1 to 7.65
333 x 10^7 ± 3.02 x 10^6 Rsed_mud genes g^{-1} FW soil/sediment. AOA ranged from 2.8 x 10^3 ± 8.63 x
334 10^2 Rsed3 to 7.69 x 10^7 ± 1.64 x 10^7 Cint genes g^{-1} FW soil/sediment (**Fig 2**). In both bays,
335 sediment AOB *amoA* gene abundances were significantly greater than AOA *amoA* gene

336 **Table 1.** Chemico-physical characteristics of chosen sites in Rusheen (R) and Clew (C) bay. DOC: dissolved organic carbon. DON:

337 dissolved organic nitrogen

Site	NO_3^- (µg N g^{-1})	NO_2^- (µg N g^{-1})	NH_4^+ (µg N g^{-1})	pH	Salinity	DOC (µg C g^{-1})	DON (µg N g^{-1})	Classification
Rsoil	1.37 (±0.13)	0.30 (±0.13)	104.88 (±21.8)	7.18 (±0.14)	5.77	82.89 (±6.22)	15.46 (±1.85)	Soil
Rint	1.71 (±0.39)	0.38 (±0.23)	119.9 (±35.57)	7.71 (±0.1)	15.34	89.02 (±18.31)	17.67 (±0.73)	Interface
Rsed_mix	1.01 (±0.08)	0.31 (±0.16)	67.45 (±7.3)	8.2 (±0.14)	30.04	72.35 (±9.11)	12.32 (±0.28)	Muddy Sand
Rsed_mud	1.51 (±0.06)	0.36 (±0.04)	82.36 (±8.01)	8.15 (±0.04)	33.01	92.55 (±3.12)	13.67 (±1.22)	Muddy
Rsed_sand	0.92 (±0.01)	0.12 (±0.04)	52.75 (±3.79)	8.52 (±0.07)	33.04	60.4 (±0.97)	10.68 (±1.01)	Sand
Csoil	2.26 (±0.09)	1.47 (±0.24)	33.55 (±8.06)	6.42 (±0.33)	4.86	77.79 (±38.53)	19.38 (±1.93)	Soil
Cint	1.46 (±0.59)	1.82 (±1.5)	118.42 (±65.73)	7.26 (±0.68)	13.11	51.61 (±5.40)	15.28 (±4.8)	Interface
Csed_mix	0.40 (±0.14)	1.04 (±0.24)	59.16 (±4.6)	8.75 (±0.03)	12.11	98.72 (±6.03)	13.82 (±0.46)	Gravelly Muddy Sand
Csed_mud	2.16 (±0.60)	2.05 (±1.52)	108.35 (±14.35)	8.13 (±0.07)	23.05	149.17 (±1.56)	24.3 (±1.96)	Muddy
Csed_sand	0.82 (±0.07)	0.25 (±0.04)	55.43 (±3.73)	8.89 (±0.12)	34.01	56.5 (±32.75)	11.15 (±3.01)	Sand

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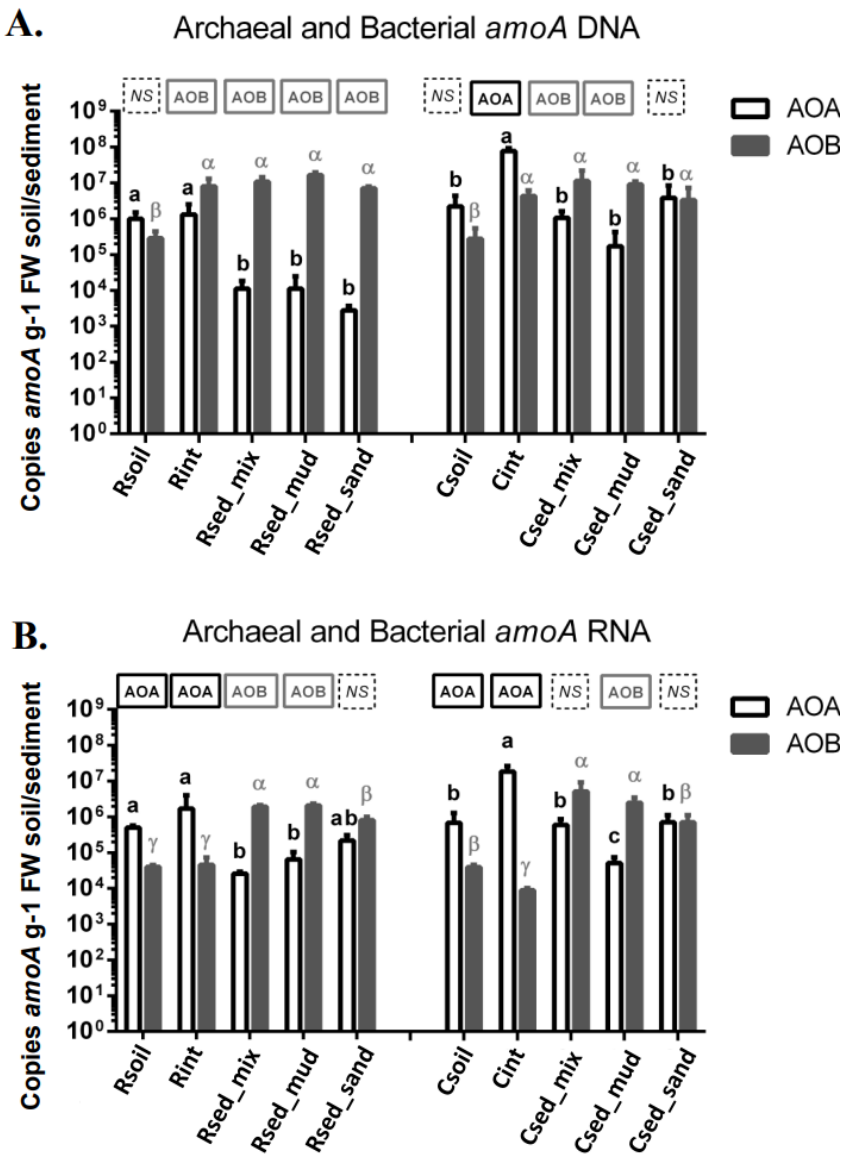
abundances at all sites except for Csed_sand ($P<0.05$). For soil sites, AOA *amoA* gene abundances were higher than AOB, although not significantly different. For the interface sites, at Rint, AOB *amoA* gene abundances were significantly greater than AOA. At Cint, AOA *amoA* gene abundances dominated and were significantly greater than those in both soil and sediment sites ($P<0.05$).

At mRNA level, *amoA* transcripts were quantified from all sites. AOB ranged from $8.87 \times 10^3 \pm 1.5 \times 10^3$ Cint1 to $5.14 \times 10^6 \pm 4.13 \times 10^6$ Csed_mix transcripts g^{-1} FW soil/sediment. AOA ranged from $1.1 \times 10^4 \pm 7.42 \times 10^3$ Rsed_mud to $7.69 \times 10^7 \pm 1.64 \times 10^7$, Cint transcripts g^{-1} FW soil/sediment **(Fig 2B**). AOB transcripts were highest in the sediments, as observed for *amoA* genes. However, there was no significant difference between AOA and AOB *amoA* gene transcript abundances among the three sediment sites (Rsed_sand, Csed_mix and Csed_sand). AOA transcripts dominated both soil and interface sites.

AOB *amoA* gene abundances in both bays were positively correlated with pH and salinity (Pearson's correlation, $P<0.05$) and with NH_4^+ in Clew Bay only (0.71, $P<0.003$) (**Tab. 2**). AOA *amoA* gene abundances in Rusheen Bay were positively correlated with NH_4^+, DON and DOC (0.853, $P<0.0003$; 0.763, $P<0.0005$; 0.747, $P<0.001$, respectively), but negatively with salinity (-0.811, $P<0.0004$). AOA *amoA* gene abundances from Clew bay did not correlate with any of the measured physical-chemical variables



357

358 **Figure 2.** Abundance of AOA and AOB *amoA* gene (A) and transcript (B) across Rusheen and
359 Clew bays. Values are mean (n=3) and error bars are standard error. One-way ANOVA and Tukey
360 post-hoc test were used to test for statistical difference in gene or transcript abundances for AOA
361 (denoted by standard letters) and AOB (denoted by Greek letter) within a bay. Within a site,
362 statistical differences in gene or transcript abundance between AOA and AOB are indicated in the
363 box on top of the bars, showing the dominant group. NS indicated no significant difference.

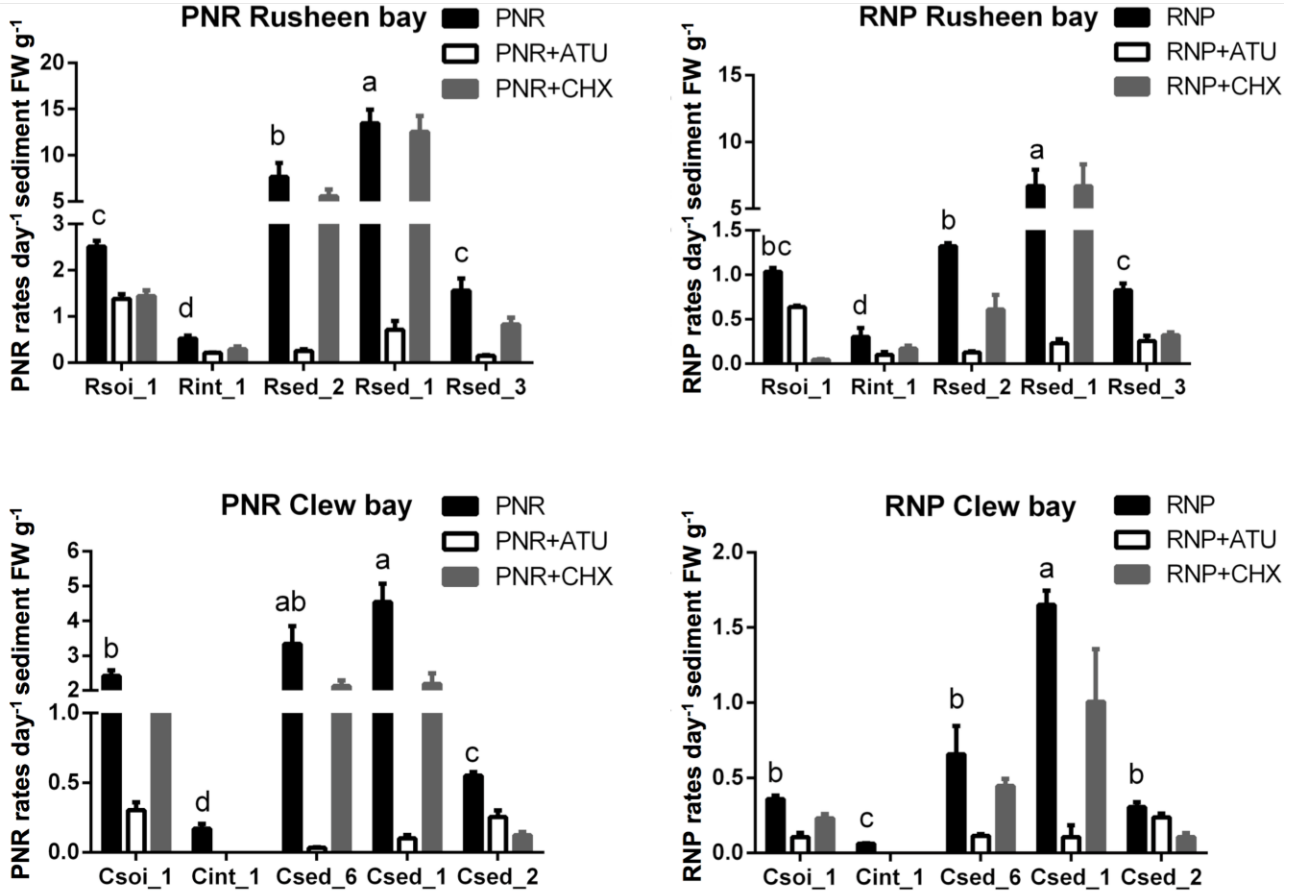
Table 2. Pearson correlations between the AOA and AOB abundance of *amoA* gene and transcripts, physico-chemical parameters and Potential Nitrification Activity (PNA) and Recovery of Nitrification Potential (RNP) in Rusheen and Clew bays. Only statistically significant results are shown, P<0.05 (*), P<0.01 (**) and P<0.001 (***). NS is non-significant.

		NO_3^-	NO_2^-	NH_4^+	pH	DON	Salinity	DOC	PNA	RNP
Rusheen bay	***amoA* AOB**	NS	NS	NS	0.569 (*)	NS	0.744 (*)	NS	0.623 (*)	NS
	***amoA* AOA**	0.627 (*)	NS	0.853 (***)	-0.797 (***)	0.763 (***)	-0.811 (***)	0.747 (**)	-0.523 (*)	NS
	***amoA* AOB RNA**	NS	NS	0.809 (***)	0.729 (*)	-0.746 (**)	0.825 (**)	-0.645 (**)	0.702 (**)	0.617 (*)
	***amoA* AOA RNA**	0.528 (*)	NS	0.532 (*)	NS	0.545 (*)	-0.574 (*)	NS	-0.719 (**)	-0.645 (*)
Clew bay	***amoA* AOB**	NS	NS	0.709 (**)	0.618 (*)	NS	0.435 (*)	NS	0.033	NS
	***amoA* AOA**	NS	NS	NS	NS	NS	NS	NS	-0.831 (***)	-0.859 (***)
	***amoA* AOB RNA**	0.555 (*)	NS	NS	0.717 (**)	NS	NS	NS	0.641 (*)	0.739 (**)

AOB transcripts from both bays had a strong positive correlation with pH (Rusheen, 0.729, P<0.002; Clew 0.722, P<0.003) (**Tab. 2**). In Clew bay, AOB transcripts were positively correlated with NO_3^- (0.555, P<0.043). In Rusheen bay, AOB transcript were positively correlated with NH_4^+, pH and salinity (but negatively correlated with DON (-0.746, P<0.001) and DOC (-0.645, P<0.0094) (**Tab. S1**). In addition, AOA transcripts were positively correlated with NO_3^-, NH_4^+ and DON. pH and salinity were positively correlated (R=0.95, P<0.05) in both Rushen and Clew bays (R=0.55, P<0.05) (**Tab. S1**).

PNA/RNP along the Gradient

In Rusheen bay, PNAs ranged from 0.7 to 14.5 day^{-1} g^{-1} FW sediment/soil (**Fig. 3A**). The highest rates were measured in Rsed_mud, while the lowest were in Rint. With the addition of inhibitors (ATU, mostly bacteria; CHX, mostly archaea), there was no significant difference between the contribution of ATU- or CHX-sensitive AO at the Rsoil and Rint sites. On the other hand, ATU-sensitive AO was significantly inhibited in the marine sites Rsed_mix, and Rsed_sand. RNP, following C_2H_2 inactivation of AMO, ranged from 0.3 to 6.1 day^{-1} g^{-1} FW sediment/soil (**Fig. 3B**), which were significantly lower than the PNAs. Highest and lowest RNPs were measured in Rsed_mud and Rint, respectively. With the addition of inhibitors, CHX-sensitive AO was significantly reduced compared to ATU-sensitive AO in Rsoil, while in Rsed_mix and Rsed_mud ATU-sensitive AO was significantly reduced. There was no significant difference between CHX and ATU-sensitive AO in Rint and Rsed_sand.



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388 **Figure 3. Potential Nitrification Activity (PNA) and Recovery of Nitrification Potential (RNP) rates across Rusheen and Clew**
389 **bay.** Values are mean (n=3) and error bars are standard error. Changes in PNA (A and C; black bars) and RNP (B and D; black
390 bars) across the bay were tested using one-way ANOVA and Tukey post hoc test. Statistically significant differences are
391 represented by standard letters on top of the bars.

In Clew bay, PNAs were between 0.25 and 4.6 day^{-1} g^{-1} FW sediment/soil, which were lower than those observed in Rusheen bay (**Fig 3C**). The highest rates were measured in Csed_mud and lowest in Cint. With the addition of inhibitors, ATU-sensitive AO was significantly reduced in Csoil, Csed_mix and Csed_mud. No significant difference was detected between CHX- and ATU-sensitive AO in Csed_sand. Despite several optimization attempts, no PNAs were measured from the Cint site when inhibitors were used. RNPs rates ranged from 0.15 to 1.6 day^{-1} g^{-1} FW sediment/soil, significantly lower compared to PNAs. When ATU and CHX were used, ATU-sensitive AO was dominant over CHX-sensitive AO in both Csed_mix and Csed_mud. No significant difference was measured in the contribution of ATU- or CHX-sensitive ammonia oxidation in Csoil and Csed_sand. Moreover, no RNPs were measured in Cint when inhibitors were used.

In Rusheen bay, PNA positively correlated with AOB *amoA* genes (0.623, $P<0.01$) and transcripts (0.702, $P<0.003$) but negatively with AOA *amoA* genes (-0.523, $P<0.045$) and transcripts (-0.719, $P<0.002$) (**Tab. 2**). While no significant correlation was observed between AOB *amoA* genes and RNP, there was a strong positive correlation at transcription level (0.617, $P<0.0018$). In contrast, AOA *amoA* transcripts negatively correlated with RNP (-0.645, $P<0.012$). In Clew bay, AOB *amoA* transcripts positively correlated with PNA (0.641, $P<0.01$) and RNP (0.739, $P<0.002$) while both AOA *amoA* genes and transcripts negatively correlated with PNA and RNP (**Tab. 2**).

PNA/RNP versus *in situ* Active Nitrifier Community Structure

Analysis of *amoA* transcript structure *in situ* and within PNA and RNP assays was carried out for soil and sediment sites in Rusheen bay only (Fig. 4). Sufficient transcripts were not recovered from

Rint or any of the Clew Bay sites for DGGE analysis. For Rsoil, only archaeal *amoA* was recovered (Fig 4A) and analysed by DGGE. While for, Rsed_mix (Fig 4B), Rsed_mud (Fig 4C) and Rsed_sand (Fig 4B), bacterial *amoA* but not archaeal *amoA* transcripts were recovered and analysed by DGGE.

In the Rsoil site for AOA (at DNA level), the *in situ* and PNA profiles were similar ($P<0.775$) and the same number of bands were detected in both ($n=11\pm1$) (**Fig. 4A**). However, at RNA level, *in situ* was significantly different to PNA ($P<0.023$), with less bands *in situ* ($n=9\pm1$) than in the PNA ($n=17\pm2$), and a Jaccard similarity of only 43%. Both PNA and RNP RNA profiles were similar ($P<0.901$) and had the same number of bands (**Fig. 4A**).

In Rsed_mix for AOB (at DNA level), *in situ* and PNA profiles were significantly different ($P<0.017$), the PNA community had 14 ± 1 distinct bands and the RNP 8 ± 2 (**Fig. 4B**). However, at RNA level, *in situ* and the PNA AOB community were similar ($P<0.389$), with a similar number of bands in both ($n=18\pm0.3$, PNA; $n=19\pm0.5$, *in situ*) and a 83% Jaccard similarity index. The transcripts from the RNP were significantly different in both community structure and number of detected bands ($n=16\pm2$, Fig 3B), to that in the PNA and *in situ*.

In Rsed_mud, for AOB at DNA level the *in situ* and PNA profiles were significantly different ($P<0.042$) with 4 ± 1 bands PNA and 2 *in situ* (**Fig. 3C**). However, at the RNA level, *in situ* and PNA profiles were far more diverse and more similar to each other ($P<0.17$), with 24 ± 1.5 *in situ* and 23 ± 2 PNA detected bands, respectively, and a Jaccard index of similarity of 86% (**Fig. 4C**). Since corresponding DNA bands were not observed in the DNA DGGE (red rectangles **Fig. 4C**), this indicates the presence of a low abundant but highly transcriptionally active group of AOB at

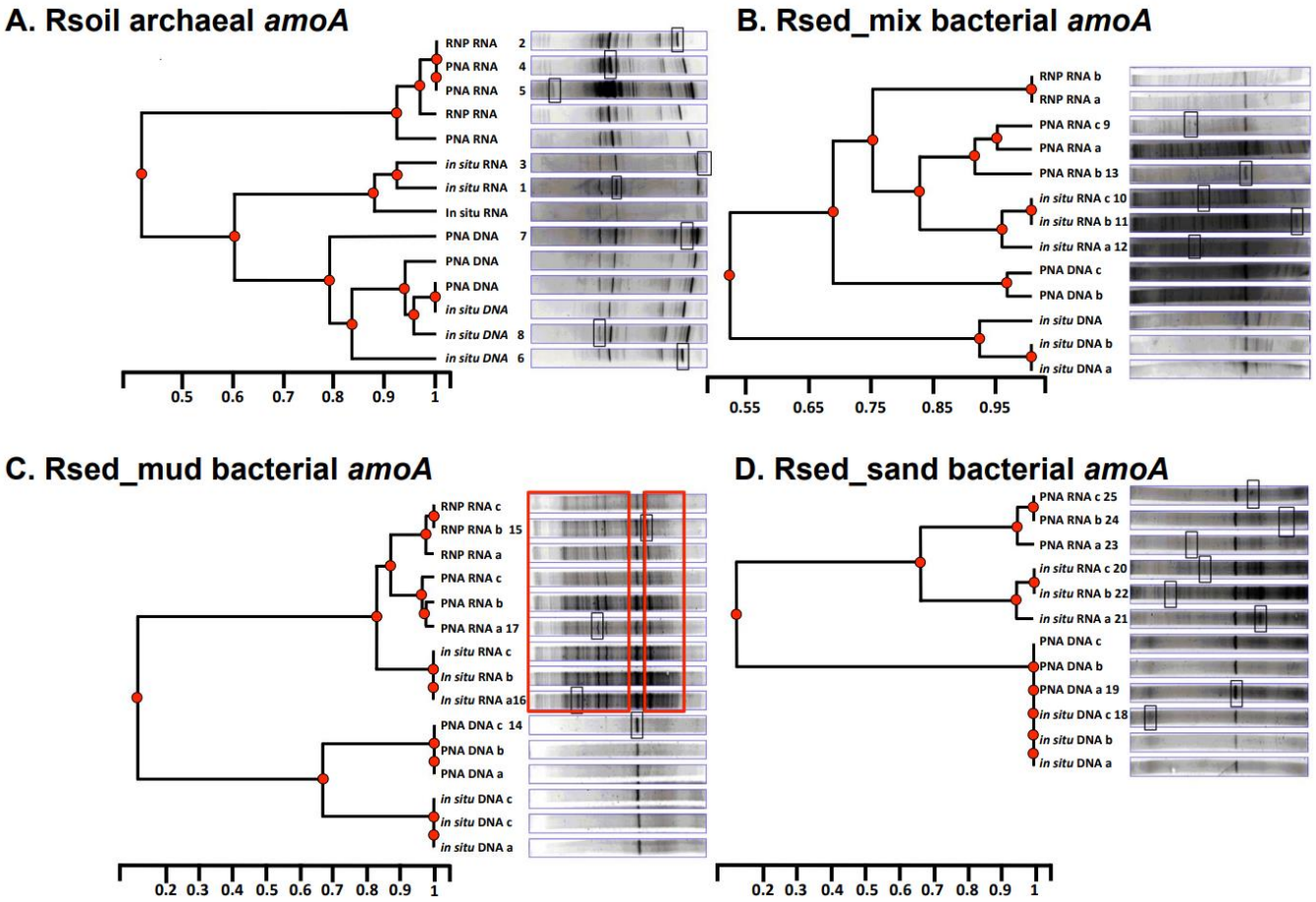

Figure 4. UPGMA-based dendrograms of DNA and RNA *amoA* community in PNA/RNP and *in situ* obtained by DGGE profiles for the analyzed sites in Rusheen bay. Bands indicated by box and number were excised and sequenced for taxonomic/phylogenetic analysis. Red rectangles in Figure 4C indicate AOB members low in abundance but highly active. Scale on the X axis represent the distance between samples (percentage dissimilarity) based on the banding pattern.

this site (**Fig 4C**). There was no significant difference (P<0.722) between the PNA and RNP RNA profiles.

In Rsed_sand for AOB (at the DNA level) the *in situ* and PNA profiles were similar (P<0.832) with two dominant bands. However, *in situ* and PNA profiles were more similar at RNA level than DNA profile, with 12±1 bands *in situ* and 8±1 on the PNA, and a Jaccard similarity of 69%. No amplification of *amoA* transcripts form RNP samples was obtained.

Some of the active ammonia oxidizers from *in situ* PNA and RNP conditions were identified following the excision and sequencing of dominant DGGE bands (Fig 4). Eight and seventeen bands were obtained from archaeal and bacterial *amoA* respectively. *amoA* sequences were generally more similar to sequence from uncultured representatives than from isolated strains (**Figs. S1** and **S2**). Bacterial *amoA* sequences grouped in a single cluster most similar to the known *Nitrosomonas* and uncultured *amoA* from similar marine/estuarine/salt marsh sediments (**Fig. S1**). The archaeal *amoA* sequences (Rsoil DGGE 1-3, Rsoil DGGE 5-8) belonged to Group I.1a along with uncultured sequences from similar sediment/soil environments (**Fig. S2**). Only one sequence, Rsoil DGGE 4 belonged to Group I.1b clustering with *amoA* sequences obtained from other soil environments.

Discussion

In the present work, we measured the abundance and transcriptional activity of ammonia oxidizers along soil-sediment gradients in two coastal bays. The sediment and soil environments showed differences in the abundance and transcriptional activity of the nitrifying communities. Gene and transcript quantification showed the dominance of AOB in sediments (**Fig. 2**), while AOA

460 transcripts were more abundant in soil and interface samples from both bays (**Fig 2**). pH and
461 salinity were key drivers separating AOA and AOB across the gradient (**Tab. 1 & 2**).

462 We further investigated the suggested trend from the molecular data on the contribution of AOA
463 and AOB to PNA/RNP using selective inhibitors. The use of inhibitors has shortcomings, with
464 some AOB insensitive to ATU (Lehtovirta-Morley et al., 2013; Shen et al., 2013) and some AOA
465 insensitive to cycloheximide (Taylor et al, 2010; Wright et al., 2020). This means that they cannot
466 be assumed to reliably inhibit only AOA or AOB. They were selected at the time of the experiment
467 as the best inhibitors, but there have since been further developments, in particular for AOA, such
468 as the use of 2-phenyl-4, 4, 5, 5, -tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (Huang et al.,
469 2021). However, use of the selected inhibitors in combination with the transcript data from this
470 work, and that of our previous work (Zhang et al., 2018), adds to the evidence and supports the
471 finding that AOA are the main contributor to AO in the soil sites while AOB are the main
472 contributor in marine sediments.

473 Exploiting this functional shift in AO, this work has shown that the experimental conditions used
474 during the measurement of nitrification potentials may lead to the response of different AO from
475 those active *in situ*, which may result in an altered view of the nitrification capability. Specifically,
476 the incubation conditions appear to be more suited to AOB than AOA. By targeting
477 transcriptionally active AOB we showed a similar community structure within the PNA/RNP
478 incubations and *in situ* for the mud and muddy-sandy sediment sites (Jaccard similarity values
479 83%, $P<0.17$ and 86%, $P<0.389$ respectively) (**Fig. 4**). Further sequencing would be required to
480 determine if these were the exact same AO. However, these findings support our hypothesis that
481 the PNA/RNP conditions did not adversely affect AOB activity and supports the high positive
482 correlations observed between AOB transcript abundance and measured PNA/RNP potentials in

from most AOB dominated sites (**Tab. 2**). In addition to the active *in situ* and PNA/RNP communities being similar for AOB, we showed that there were more DGGE bands in the RNA compared to DNA and a clear separate clustering of DNA and RNA (**Fig. 4C**). This indicates that there are members within the AOB community that are low in abundance (DNA DGGE) but highly active both *in situ* and in the PNA/RNA experiments (**Fig 4**). This result is consistent with Zhang et al. (2018) who found a higher number of bands in the RNA DGGE compared to DNA DGGE and with Duff et al. (2017) who found AOB OTUs present in cDNA but not DNA clone libraries from muddy sediments. In this study, the sequencing of bands that were found only in the RNA revealed that they were affiliated to unknown *β*-proteobacteria. Future studies should focus on identifying these unknown transcriptionally active AOB that likely drive ammonia oxidation in sediments, for example though DNA/RNA SIP to label and target active nitrifiers.

For AOA we showed that the incubation conditions did not select for the same active AOA community as *in situ*, with a low level of similarity (43%) between active AOA *in situ* and PNA/RNP community structure for the soil site (**Fig. 4)**. For all soil and interphase sites, AOA transcripts were greater than AOB (**Fig. 2**), yet the most striking example of inconsistencies between transcripts abundance and potential rates was observed at the interphase soil/sediment. At these sites the highest AOA *amoA* transcripts were recorded but no PNA/RNP was measured despite extensive optimization efforts. Previously we have shown that both AOA and AOB were contributing to nitrification in the soil/sediment interface (Zhang et al., 2018), and this may explain why it was difficult to optimize PNA for both groups. Other authors have indicated transition zones between terrestrial and aquatic ecosystems as hot-spots for biogeochemical processes, since they are crossed by both terrestrial and marine fluxes of nutrients, in particular the oxidized and reduced forms of N (McClain et al., 2003; Zhu et al., 2013). Further investigations in different locations

are needed to clarify nitrification dynamics in interface sites between terrestrial and marine environments. Indeed, at sites the use of RT-Q-PCR was a better indicator of AO activity than the PNA/RNP, with the added advantage of the ability to distinguish between AOA and AOB.

Although an overall negative correlation was observed between AOA transcript abundance and PNA/RNP (**Tab. 2),** rather than conclude that AOA are not active in the soil/interphase *is situ*, we suggest that standard PNA/RNP incubation conditions are not favoring the resident active AOA. This could be due to the excess of inorganic ammonia, O_2, agitation or homogenization used in the assays. Further the AO from these sites may prefer other N sources rather than NH_4^+ provided, for example from cyanate (Palatinszky et al., 2015). Indeed, investigations of niche separation in soil have shown the preferential activity of AOA when ammonia is supplied via mineralisation of organic matter (Aigle et al., 2020). In this study, we found a positive and significant correlation between AOA *amoA* transcript abundance and DON in Rusheen bay (**Tab. 2**) which could indicate that AOA may use DON-derived ammonia in this environment. A recent review by Hazard et al. (2021) showed that of 107 studies only 36% reported a significant positive correlation between PNA and AOA gene abundance in soils and sediments verses 75% of studies showing a positive correlation for AOB. They argued that instead of concluding that AOA is not active, as if often the case, this low correlation is due to the inappropriate PNA incubation conditions that will differently affect AOA and AOB. Indeed, PNA is not only affected by nitrifier abundances but also by community composition (Hazard et al., 2021) which implies that physiological diversity within communities will require bespoke incubation conditions for different groups of AO. This work supports those conclusions.

Conclusions

Our study investigated the distribution, activity and dominance of AO across transition zones between terrestrial (soil) and marine (sediment) environments. Gene and transcript data indicated that AOA dominated in soil and interphase and AOB dominated in marine muddy sediments. For AOB, a higher correlation was observed between PNA/RNP and transcript abundance compared to DNA. Furthermore, RNA DGGE revealed the presence of active AOB *in situ* and in the PNA experiment that were of too low abundance to be detected in the DNA DGGE. Interestingly, we show that PNA/RNP analyses may provide a good picture of the environmental nitrification capacity when AOB are the dominant group, with the same transcripts present at the end of PNA/RNP incubation and detected *in situ*. On the other hand, negative correlations between AOA transcript abundance and PNA/RNP were observed, indicating that incubation conditions used in this study did not favor the resident active AOA. This supports our hypothesis that PNA is suited to reflect AOB but not AOA activity.

Declaration of competing interest

We have no conflicts of interest to declare.

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Credit Authorship Contribution Statement

ET: Conceptualization; Investigation, Methodology; Writing - original draft; **AMD**: Conceptualization, Writing - review & editing. **AK** and **FC**: Writing – review and editing; **UZI**: Visualization, Software; **CJS** Conceptualization; Methodology, Writing; Supervision, Resources, Funding acquisition.

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